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THE AUTOEPIOTOPE OF THE 74-kD MITOCHONDRIAL AUTOANTIGEN OF PRIMARY BILIARY CIRRHOSIS CORRESPONDS TO THE FUNCTIONAL SITE OF DIHYDROLIPOAMIDE ACETYLTRANSFERASE

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Primary biliary cirrhosis (PBC)¹ is a chronic idiopathic autoimmune liver disease characterized by the specific destruction and obliteration of the intrahepatic bile ducts (1). The major serologic feature of this disease is the production of anti-mitochondrial antibodies (AMA) by 90–95% of PBC patients (2, 3). Recently, our laboratory reported the cloning and sequence of a cDNA from rat liver that encodes for the major mitochondrial autoantigen, a 74-kD protein (4). This latter protein is found in the inner mitochondrial membrane and is now identified as part of the core protein, E2, of the pyruvate dehydrogenase complex (PDH) (5). Indeed, the sequence of the recombinant mitochondrial protein shows significant homology to a published sequence of PDH-E2 in *Escherichia coli* (6). PDH consists of three component enzymes, termed E1, E2, and E3. E1 is a thiamin pyrophosphate-dependent 2-oxo acid dehydrogenase, and E3 is an FAD-dependent lipoamide dehydrogenase. E2 is an acyltransferase that forms the central core of the complexes and uses lipoic acid, which is covalently bound to the epsilon amino group of lysine, as an essential cofactor (7). Antibodies to PDH-E2 are not found in patients with other chronic autoimmune liver diseases. It was reasoned that while identification of the molecule PDH-E2 as a specific autoantigen for PBC provides an important diagnostic tool, the precise definition of the epitope(s) within this molecule may provide important insights into the molecular basis of self-tolerance. These thoughts prompted us to use our 1370-bp cDNA clone (4), which contains and expresses PDH-E2, to determine the immunodominant epitope. To characterize the region that contains the epitope, we digested pRMIT with restriction endonucleases and subcloned the resulting fragments into frame-shifted expression vectors. These studies led to the

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¹ *Abbreviations used in this paper:* AMA, anti-mitochondrial antibodies; HEL, hen egg lysozyme; IPTG, isopropyl-thiogalactosidase; PBC, primary biliary cirrhosis; PDH, pyruvate dehydrogenase complex; RT, room temperature.

identification of a cDNA clone, which is designated pRMIT-603, located between bases 76 and 679 of pRMIT, and codes for a peptide that contains the epitope recognized by sera from PBC patients. Generation of synthetic peptides encompassing distinct hydrophilic peaks within this region led to the identification of one 20-amino acid peptide that corresponded to the lipoate binding site of the PDH-E2 molecule (6). This peptide, containing residues 81-100, absorbed autoreactivity to the original clone in an inhibition assay, suggesting that the autoimmune response against PDH-E2 corresponds to this lipoate binding site. The identification of the precise amino acid sequence of this ubiquitous self protein provides a powerful model for the delineation of the mechanisms involved in the breakdown of self-tolerance.

Materials and Methods

Sera. Sera were collected from 22 patients having a well-established clinical and laboratory diagnosis of PBC. In addition, sera from 20 healthy volunteers were used as normal controls. All test and control sera were from the clinics of the University of California at Davis and the Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia.

cDNA Clone and Restriction Digestion. A 1370-bp cDNA clone was isolated from a rat liver cDNA λ gt-11 Amp³ library as previously described (4). The nucleotide sequence of the cDNA insert was determined, the restriction sites were mapped, and the correct frame was determined by double-stranded sequencing of an expressing plasmid clone designated pRMIT (4, 8, 9). To identify regions of the insert that encoded for reactive polypeptide recognized by sera from patients with PBC, restriction enzyme digests were performed (see below). The fragments were isolated on 1% low melting point agarose and religated into one of the pUR series of vectors chosen to retain in-frame expression (10). Successful ligation was confirmed by colony hybridization with ³²P-dCTP nick-translated pRMIT. Thus, the 1370-bp clone in pBTA 224 was digested with Hind III, which cuts the insert once at restriction site 915 and the plasmid once at a site 3' of the Eco R1 insertion site. This digestion generates a large fragment containing the plasmid and a portion of the insert, and a smaller fragment of 455 bp at the 5' end of the insert (Fig. 1). The plasmid-containing fragment was re-ligated to itself and the small fragment reinserted into pBTA 224 at the Hind III cloning site for expression. The original 1370-bp insert was also cut with Pst I to generate six fragments (Fig. 1).

Expression of Fragments in JM101. Duplicate arrays of the above recombinant clones were tested for expression of protein recognized by PBC sera by first inducing expression with 100 μ g/ml isopropyl-thiogalactoside (IPTG) for 4 h at 37°C. Colonies were prepared for antibody probing as described (11). Briefly, colonies were lysed with 1% SDS in a chloroform atmosphere for 30 min, washed with 3% milk in PBS, and blocked with milk PBS for 15 min. The sera used for probing were previously absorbed with lysates of wild-type *E. coli* K12 JM101. Filters containing *E. coli* K12 JM101 colonies were probed with a 1:1,000 dilution of PBC sera for 45 min, were washed three times, and were incubated with either I¹²⁵ goat anti-human Ig or goat anti-human total Ig peroxidase conjugate (TAGO, Burlingame, CA) for 1 h. To visualize reactivity, 0.6 mg/ml 4-chloro-1-naphthol in 20% methanol/PBS and 0.03% hydrogen peroxide were added for 10 min; the reaction was stopped with distilled H₂O.

Immunoblots. 100 ml of overnight cultures of *E. Coli* K21 JM101 cells transformed with either positive or negative clones were diluted 1:10 in Luria-Bertani broth containing 10 mM IPTG. 4 h later, the culture was centrifuged at 5,000 *g* for 10 min then snap frozen after the addition of 20 ml of PBS. PAGE was performed on 1-mm thick slab gels in 0.1% SDS using a 3.8% stacking gel and a 10% resolving gel. Samples were diluted 1:100 in sample buffer containing 5% 2-ME in 3% SDS and, were boiled for 5 min. Each lane contained 5-10 μ g of protein. The samples were probed with PBC sera as described, and reactivity was determined by enzyme immunoassay (9).

Isolation of Fused Polypeptide for Absorption. Absorption of reactivity from PBC sera was performed using the purified expression product of pBTA containing the 603-bp insert. pRMIT-

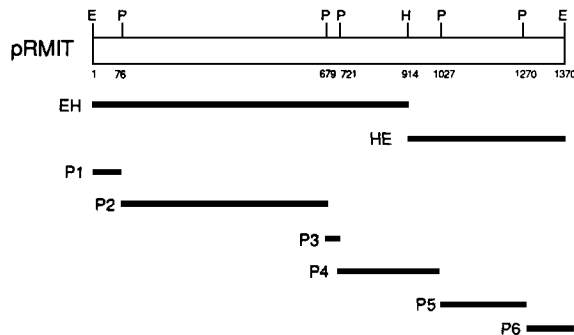


FIGURE 1. Restriction digestion map of pRMIT. The flanking Eco RI (*E*) sites are found at the 5' and 3' ends of the original clone. The fragments produced by Hind III digestion are labeled *EH* (5') and *HE* (3'). The Pst I-generated fragments are labeled *P1* (5')–*P6* (3'). pRMIT-603 corresponds to fragment *P2*. All of the above fragments were ligated to the appropriate frame shifted vectors for expression and assay.

603 was incubated overnight at 37°C in Luria-Bertani broth containing 25 µg/ml ampicillin, diluted to log-phase growth, and induced with 10 mM IPTG for 4 h at 37°C. The cells were then harvested at 5,000 *g* for 10 min, and the pellets were resuspended in 40 ml of 10 mM Tris-HCl, pH 8.0, containing 2 mM EDTA. Lysozyme was then added to a final concentration of 0.2 mg/ml, and the mixture was rotated at room temperature (RT) for 30 min. The solution was then brought up to 0.2% Triton-X 100 with continuous mixing for 10 min at RT. An equal volume of 10 mM Tris-HCl with 2 mM EDTA, 50 mM NaCl, and 10 mM MgCl₂ was added containing a final concentration of 2 mg/ml DNase. This mixture was rotated for 14 min at RT then spun at 1,400 *g* for 5 min. The pellet was discarded and the supernatant was spun for 30 min at 10,000 *g*. This final pellet was dispersed in 0.1 M phosphate buffer, pH 6.0, with 2% SDS and 10 mM dithiothreitol, and was fractionated on a Sephacryl S300 column in tandem with an S400 column. The column was eluted at 50 ml/h and 6-min fractions were collected for assay by analytical SDS-PAGE and immunoblotting (4).

Absorption. Antibodies were absorbed two times by incubating PBC sera, diluted 1:100, with 40 µg/ml of purified pRMIT-603 in 2% milk PBS overnight at 4°C on a rocker and centrifuged at 5,000 *g* for 10 min. An unrelated recombinant purified fusion protein, the rat F liver antigen, was used as an absorption control (12). Sera from 22 different patients with PBC were then diluted 1:1,000, 1:10,000, and 1:100,000 and assayed by immunoblotting against purified placental mitochondria as described above.

Peptide Synthesis and ELISA. Based upon hydrophobicity/hydrophilicity predictions of antigenic regions within the 603-bp clone, six peptides were synthesized. These were residues 64–83, 81–100, 105–114, 121–140, 188–203, and 201–221 (Fig. 2). Peptides corresponding to

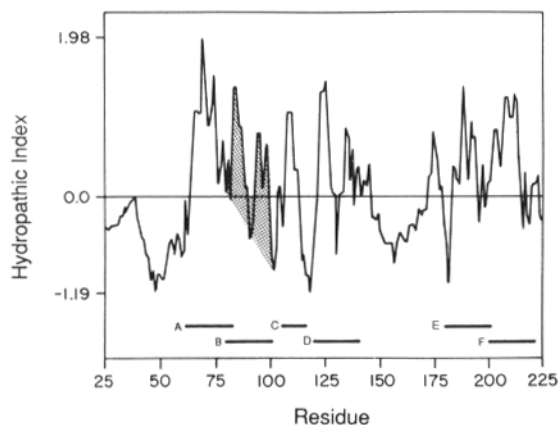


FIGURE 2. Hydrophilicity plot of pRMIT [Hopp and Woods] (13). The plot of hydrophilic regions (*above baseline*) vs. amino acid residues of pRMIT show several regions of potential antigenicity. The synthesized peptides are (A) 64–83, (B) 81–100, (C) 105–114, (D) 121–140, (E) 188–203, and (F) 201–221. The reactive peptide (B), designated by the shaded area between residues 81–100, contains two distinct hydrophilic peaks joined by a hydrophobic region.

TABLE I
Amino Acid Sequences of Synthetic Peptides Tested for Absorption

Residue	Sequence
64-83	QRWEKKVGEKLSEGDLLAEI
81-100	AEIETDKATIGFEVQEEGYL
105-114	VPEGTRDVPL
121-140	IVEKQEDIAAFADYRPTEVT
188-203	LAAEKGIDLTQVKG
201-221	GTGPEGRIKKDIDSFVPTKA

Six peptides were synthesized corresponding to the major hydrophilic peaks contained in pRMIT-603.

these residues were synthesized on a synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) using Merrifield Boc polystyrene peptide synthesis chemistry (Table I). The resulting peptides were purified on an HPLC apparatus (Beckman Systems, Fullerton, CA).

An inhibition ELISA was implemented to determine the putative clone or synthesized peptide fragments that would competitively inhibit the binding of PBC sera to the original reactive peptide. Briefly, polystyrene microtiter plates were coated with the purified polypeptide expressed by pRMIT at 2.5 µg/ml overnight at 4°C. The plates were washed three times with 0.05% PBS Tween and were blocked with 1% BSA/PBS. Serial dilutions of patients' sera in a volume of 0.1 ml from 1:1,000 to 1:100,000 were incubated with the mitochondrial-purified fusion polypeptide (34 µg/ml), the purified F antigen fusion polypeptide (50 µg/ml), or synthetic peptide (100 µg/ml) for 4 h at RT. The absorbed sera were incubated on the blocked plate in tandem with unabsorbed sera for 1 h. After washing, the plates were incubated with 0.1 ml of a goat anti-human peroxidase conjugate diluted 1:4,000 (TAGO, Burlingame, CA) for 30 min. Reactivity was visualized with 40.0 mM 2,2'-Azinobis (3-ethyl benzthiazoline sulfonic acid) (Sigma Chemical Co., St. Louis, MO) in 0.05 M citric acid buffer, pH 4.2, containing 0.05 M H₂O₂.

Results

Restriction Enzyme Digestion. Hind III digestion of the insert yielded two fragments; immunological reactivity was confined only to the EH clone, i.e., the 5' fragment (nucleotides 1-940) (data not shown). Digestion with Pst I yielded six fragments and only clone P2 (nucleotides 76-679) was reactive with PBC antibody. Reactivity could be demonstrated in colony immunoassay (data not shown) as well as by Western blotting of the β-galactosidase fusion protein produced by *E. coli* induced with IPTG (Fig. 3). None of the other Pst I subclones were reactive with patient sera. Control sera were unreactive with all subclones tested.

Specificity of the Fused Polypeptide. To determine whether the clone pRMIT-603 contains all of the antigenicity ascribed to the original 1,370-bp clone pRMIT, sera from 22 PBC patients were absorbed with the purified protein product of pRMIT-603 and then used to probe beef heart mitochondria in a Western blot. After absorption, reactivity was no longer seen against the 74-kD autoantigen, or dihydrolipoamide transferase, in sera from 22/22 patients. In contrast, reactivity to the 52-kD mitochondrial band was unaltered. Furthermore, absorption with the irrelevant recombinant F alloantigen fusion polypeptide did not remove any reactivity to either the 74-kD or the 52-kD protein (Fig. 3). Similar results were observed using an ELISA with the original fusion polypeptide as antigen. Sera from all 22 patients completely lost reactivity to the PDH complex after absorption. For example, the mean pre-

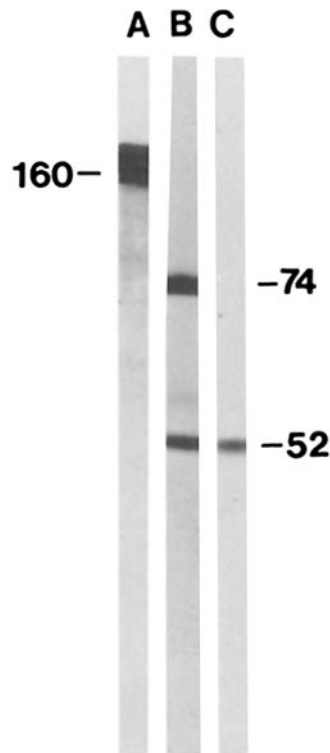


FIGURE 3. Specificity of the fused polypeptide. (A) The purified 160-kD fusion protein produced by pRMIT-603 was probed with PBC sera at a dilution of 1:1,000. (B) Beef heart mitochondria was probed with PBC serum at 1:1,000, pre-absorbed for 72 h with an irrelevant fusion polypeptide, the rat F liver antigen. (C) Beef heart mitochondria was probed with the same serum, at 1:1,000, after absorption for 72 h with the purified fusion polypeptide produced by pRMIT-603. Note that reactivity to the 74-kD band, or dihydrolipoamide acetyltransferase, of beef heart mitochondria was specifically eliminated upon absorption with the purified recombinant mitochondrial autoantigen produced by pRMIT-603.

absorbed sera OD at a 1:1,000 dilution was 1.61 ± 0.208 (SD) compared with a mean OD for absorbed sera at an equal dilution of 0.202 ± 0.233 (Table II). Upon absorption of the sera with the six synthetic peptides, reactivity of PBC sera to pRMIT was reduced only by residues 81-100. For instance, at a dilution of 1:80,000, the mean OD for the absorbed sera was 0.097 ± 0.075 compared with the unabsorbed OD of 0.812 ± 0.010 (Table III). This peptide encompasses two hydrophilic peaks joined by a slightly hydrophobic region of four amino acids.

TABLE II
Specificity of the Fused Polypeptide as Determined by ELISA

Group	Optical density*		
	Before absorption	Absorbed with pRMIT-603	Absorbed with pRFA
	mean \pm SD	mean \pm SD	mean \pm SD
PBC (n = 22)	1.61 ± 0.208	$0.202 \pm 0.233^\dagger$	1.42 ± 0.219
Control (n = 5)	0.189 ± 0.053	0.151 ± 0.045	0.165 ± 0.049

Sera from 22 patients defined as PBC patients were used at a dilution of 1:1,000 and five normal controls were diluted to 1:1,000. Aliquots were assayed before and after absorption with pRMIT-603 as described in Materials and Methods.

* 492 nm.

$^\dagger p \leq 0.001$, Student's *t* test, compared with pre-absorption and pRFA groups.

TABLE III
Absorption of PBC Sera with Synthetic Peptides as Determined by ELISA

Peptide	Optical density*	
	Pre-absorbed (<i>n</i> = 10)	Absorbed (<i>n</i> = 10)
	<i>mean</i> ± <i>SD</i>	
64-83	0.812 ± 0.010	0.825 ± 0.026
81-100	0.812 ± 0.010	0.097 ± 0.075†
105-114	0.812 ± 0.010	0.815 ± 0.005
121-140	0.812 ± 0.010	0.797 ± 0.019
188-203	0.812 ± 0.010	0.832 ± 0.015
201-221	0.812 ± 0.010	0.778 ± 0.014

Synthetic peptides were made corresponding to hydrophilic peaks contained in pRMIT-603 and tested in a competitive inhibition ELISA with PBC sera at a dilution of 1:80,000.

* 492 nm.

† *p* ≤ 0.001, Student's *t* test, compared with other peptides.

Discussion

The process by which self proteins are presented as antigens in the generation of an autoimmune response is poorly understood. In addition, the mechanism by which ubiquitous intracellular mitochondrial proteins become antigenic provides still another dimension of complexity. Work done with hybridomas and synthetic peptides has proven that virtually any portion of a protein, under various conditions, is capable of producing an antibody response (14, 15). Therefore, it is important to make the distinction between major antigenic sites, and those simply capable of generating an immune response under controlled circumstances.

It has become apparent from the study of several model antigens such as myoglobin, lysozyme, and cytochrome *c* that major epitopes are topographic structures located on the surface of a protein (14-16). These immunodominant determinants bind a greater proportion of the antibodies produced in a normal immune response against a native protein antigen than do other surface areas. Such topographic determinants may be contained within a single segment of amino acid sequence, but not necessarily involving continuous residues in the segment, or they may be assembled from residues far apart in the primary sequence but brought together on the surface by tertiary structure. One major feature of immunodominant epitopes is that they usually contain a preponderance of charged and polar amino acids, thus making them hydrophilic in nature (16). There are several algorithms that calculate average hydrophilicity and secondary structures of short segments of amino acid residues within a longer sequence (17, 18).

Such analysis has been performed on the La and Sm antigen found in SLE (19, 20) and the thyroid antigens of autoimmune thyroiditis (21). In the case of the La antigen, using analysis based on the Hopp and Woods scale, the precise location for the La determinant has identified a strongly hydrophilic decapeptide within a defined region (19). In a previous report, we described a rat liver cDNA clone designated pRMIT, which consists of a 1,370-bp fragment that codes for the 74-kD mitochondrial autoantigen of PBC (4). Similar predictions based on hydrophilicity

TABLE IV
Amino Acid Sequences of Flanking Lipoyl-Lysine Residues for
Lipoate-containing Proteins

Source	Enzyme	Sequence
pRMIT-603 (residues 83-92)	PDH-E2	Ile- Glu-Thr-Asp-Lys-Ala-Thr -Ile- Gly-Phe
Bovine heart (22)	PDH-E2	Val- Glu-Thr-Asp-Lys-Ala-Thr -Val- Gly-Phe
		and Lys-Ala-Thr-Ile- Gly-Phe
<i>E. Coli</i> (6)	PDH-E2	Val- Glu -Gly- Asp-Lys-Ala -Ser- Met-Glu-Val

Significant homology is seen between pRMIT-603 residues 83-92 and bovine heart PDH-E2, as indicated by boxed regions.

using Hopp and Woods analysis (18) for pRMIT show several possible antigenic sites: peaks at peptide residues 64-83, 81-100, 105-115, 120-140, 188-203, 201-221, and 368-382. These predictions allowed us to target our restriction analysis to particular regions of the 1,370-bp sequence. For example, the clone pRMIT-603, which contains residues 64-83, 81-100, 105-115, 120-140, and 188-203, was able to absorb 74-kD reactivity in all 22 sera tested. In addition, the complete absorption of the 74-kD, but not the 52-kD, protein reactivity with purified pRMIT-603 suggests that the major epitope is confined to this 200 residue region for the 74-kD protein in AMA. Moreover, since reactivity is retained using antigen in a reducing SDS-PAGE, it is likely that antigenicity is not completely dependent on the tertiary structure of the fusion protein. The synthetic peptide 81-100 absorbs nearly all reactivity from PBC sera but not until the sera were diluted to 1:80,000. These findings may be attributed to the properties of synthetic peptides vs. whole molecules as was demonstrated by Benjamin et al. (14). It has been noted that purified peptides for hen egg white lysozyme (HEL) react with monoclonal and polyclonal antibodies with far lower affinity than native HEL. Residues outside the 81-100 region may also contribute to a particular conformation or form part of an assembled topographic determinant.

The entire mammalian E2 sequence of PDH and the amino acids surrounding the lipoate-attachment site in bovine heart PDH complex have been recently identified (6, 22) and have been labeled A1_a, A1_b and B1_a, B1_b. When the amino acid sequence of pRMIT was compared with that of the above peptides, there was complete homology between B1_a, B1_b and a hexapeptide contained in residues 81-100. Moreover, except for two Val-Ile substitutions, A1_a, A1_b also shows complete homology with the decapeptide surrounding the Lys at residue 87 (Table IV). Interestingly, a significant amount of homology also exists between the PDH lipoyl-lysine residues of *E. coli* and the pRMIT-603 Lys region (Table IV) (6). The lipoic acid attachment site found on native E2 may play a role in the recognition of PBC autoantibodies. If, in fact, the fusion protein does have lipoic acid attached by the *E. coli* host during

production, that would account for the ability of the fusion protein to absorb out all AMA reactivity at a low serum dilution while there is little effect produced by the synthetic peptide 81-100 until the sera is diluted 80-fold.

The role of autoantigens in the ontogeny and pathogenesis of PBC is unclear. It is possible that PBC anti-mitochondrial antibodies are merely a response to injury. Evidence in favor of a primary role for these autoantibodies in the disease process is seen in both the high circulating antibody titers ($1:10^6$) and the specificity of these autoantibodies against a functional site on the target enzyme dihydrolipoamide acetyltransferase. However, regardless of their role in the disease process, the distinction between self and foreign antigens is an important issue. Moreover, the possibility of crossreactivity between the mitochondrial epitope and an etiological agent does exist, especially when one considers the degree of homology between the *E. coli* lipoyl-lysine residue and peptide 81-100. Only through the complete definition of this epitope can we approach these issues.

Summary

Autoantibodies to mitochondrial antigens are characteristic of the autoimmune liver disease primary biliary cirrhosis (PBC), but the precise antigenic determinants recognized by these antibodies have not been defined. Recently, our laboratory identified a 1,370-bp rat liver cDNA clone that coded for a polypeptide recognized specifically by sera from patients with PBC but not by sera from patients with other forms of liver disease. This recombinant protein was identified as the 74-kD M2 mitochondrial inner membrane autoantigen, now known to be dihydrolipoamide acetyltransferase. In the present study, we have identified a 603-bp fragment that codes for a polypeptide containing all of the autoreactivity of the original clone. In addition, based on hydrophobicity/hydrophilicity plots of the amino acid sequence of this polypeptide segment, several peptides were synthesized and tested for reactivity by an inhibition assay using sera from patients with PBC. One peptide, defined by the amino acids AEIETDKATIGFEVQEEGYL, absorbed serum reactivity to the protein product of the original clone. Of particular interest was the finding that this peptide contains the lipoic acid binding site KATIGF of the dihydrolipoamide acetyltransferase found in the inner mitochondrial membrane. Thus, it appears that for this autoantigen, the target of the autoantibodies corresponds to a functional site of the dihydrolipoamide acetyltransferase.

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